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BEHAVIORAL, ELECTROPHYSIOLOGICAL, AND MORPHOLOGICAL INVESTIGATIONS OF STATOCYST FUNCTION IN THE NUDIBRANCH MOLLUSC *HERMISSENDA CRASSICORNIS*

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Abstract

Laboratory-reared specimens of the nudibranch *Hermissenda crassicornis* (Eschscholtz, 1831) possess statocysts that contain either one, several, or a species-typical number of statoconia (150–200). The number of statoconia per statocyst is significantly related to the growth rate of the animal during its first month post-metamorphosis, with slower-growing individuals (<0.88 mm/day) tending to possess a single statoconium bilaterally. Laboratory-reared animals with statocysts containing a single statoconium exhibited significantly weaker conditioning effects as expressed by faster response latencies to enter an illuminated area one and two days following behavioral training as compared with laboratory-reared animals with more than one statoconium per statocyst. Rotation of the isolated nervous systems of preparations with a single statoconium revealed that caudal hair cells exhibited reduced depolarization and the hyperpolarization of Type B photoreceptors in response to rotation was absent. As the number of statoconia increased, caudal hair cell depolarization and type B cell hyperpolarization occurred with increasing frequency. Cell responses to rotation were identical to those recorded from wild-type cells when the number of statoconia exceeded eight.

Statocysts from laboratory-reared animals with a single statoconium possess the same number (13) of sensory (hair) cells, but differ from typical statocysts (150–200 statoconia) in that they are about half the normal size, the lumenal diameter is reduced, the hair cells are about twice as thick, and the sensory cilia are closer together. Both ciliary morphology and basic histology are similar in laboratory-reared and field-collected animals.

Decreased mechanotransduction is postulated to be primarily responsible for the changes in behavioral conditioning and sensory cell activity observed in these laboratory-reared animals. This decreased sensory transduction results from reduction in statoconia number and restricted movement of the stones in statocysts with reduced lumina.

Introduction

The paired statocysts of the Pacific Coast nudibranch mollusc *Hermissenda crassicornis* (Eschscholtz, 1831) are spherical fluid-filled structures containing a mass of

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discrete stones (statoconia), which interact with the tips of cilia projecting from the sensory (hair) cells lining the lumen (Alkon, 1975; Grossman *et al.*, 1979; Kuzirian *et al.* 1981). Statocysts of this type, common in gastropod molluscs, are generally considered to be mechanoreceptors (Wolff, 1975). Intracellular recordings from hair cells during statocyst displacement or during rotation of the isolated nervous system, have shown *Hermissenda* statocysts to be sensitive to an increase in gravitational force (Alkon and Bak, 1973; Alkon, 1975; Tabata and Alkon, 1982; Goh and Alkon, 1984). Electrophysiological analysis of the functional inter-relationships between the visual and mechanoreceptive pathways has been facilitated by the relatively small number of neurons comprising these two sensory networks (Alkon, 1974a; see review by Alkon, 1980). These two sensory systems are important behaviorally since a conditioning procedure consisting of illumination paired with rotation results in a long-term modification of phototactic behavior in *Hermissenda* (Alkon, 1974b; Crow and Alkon, 1978; Farley and Alkon, 1982; Crow and Offenbach, 1983; Crow, 1983, 1985; Harrigan and Alkon, 1985).

Hermissenda was reared in the laboratory under defined environmental conditions (Harrigan and Alkon, 1978) to provide animals of known history for similar behavioral and neurophysiological studies. Crow and Harrigan (1979) found that laboratory-reared animals showed significantly less behavioral variability in response to light before training, and in some cases following the training procedure. Dissection of these laboratory-reared animals revealed variation within and between individuals in the number of statoconia per statocyst, ranging from one to the typical complement of 150–200 per statocyst. Deformed or absent statoliths in hatchlings of eight cephalopod species that underwent embryonic development in artificial seawater in the laboratory have been reported (Colmers *et al.*, 1984), and veliger larvae of the opisthobranch mollusc *Aplysia californica* have been shown to require the addition of the element strontium to artificial seawater for normal shell and statolith development prior to hatching (Bidwell *et al.*, 1986). In our study, *Hermissenda* larvae cultured in natural sea water possessed normal shells and one statoconium per statocyst; morphological variations in statocysts were observed only in post-metamorphic animals and were correlated with antibiotic treatment (Crow and Harrigan, 1979).

Alkon (1975), Grossman *et al.* (1979), Grossman and Alkon (1983), and Stommel *et al.* (1980), in separate investigations of sensory transduction in the statocyst, concluded that hair cell voltage noise and generator potentials arise in response to the force exerted on the hair cell sensory cilia by the weight of the mass of statoconia. In those studies, occasionally one of the paired statocysts from a field-collected *Hermissenda* was found to contain a single statoconium. The baseline hair cell noise was reduced in these animals (Grossman *et al.*, 1979).

Crow and Harrigan (1979) suggested that because animals with a single statoconium appeared to be less affected by training with paired stimulation, the study of laboratory-reared animals with variable numbers of statoconia would aid in clarifying the role of the statocyst in mediating the behavioral change produced by conditioning. In the present study we will first describe the relationship between laboratory culture conditions, growth rates of the animals, as well as the morphology of the adult statocyst, including number of statoconia. Next, we will present behavioral, morphological, and neurophysiological evidence from these same animals which demonstrates a relationship between the presence of a single statoconium, diminished effectiveness of the conditioning procedures, and reduction in the size of the hair cell generator potential. Changes in photoreceptor responses to rotation in the case of the single statoconium are also described.

Materials and Methods

Culture conditions

Wild *Hermissenda* were provided by M. Morris, Sea Life Supply, Sand City, California. Animals were maintained in individual containers in a flow-through seawater system at 13°–15°C. Initially, egg masses were obtained from parents within one week of arrival in the laboratory and incubated until hatching at 14° ± 2°C under a 12 hour light/dark cycle in 200 ml of 0.22 μm-filtered seawater, changed daily.

Adults were isolated upon arrival in the laboratory and the first egg masses deposited by each were discarded to determine the condition of the parents' statocysts relative to those of the offspring. Previous observations (Harrigan) showed that animals isolated on arrival in the laboratory, usually 3–5 days after collection, tended to deposit one large fertile egg mass within 4 days after isolation. Subsequent egg masses laid by isolates had a low percent fertilization and were often non-viable. Rutowski (1983) found that individual *Hermissenda* isolated after copulation store enough sperm to fertilize 2–3 egg masses; physiological stresses associated with collection and shipment may have reduced gamete viability or stimulated production of especially large egg masses after isolation in our laboratory, resulting in relative sperm depletion after the first egg mass was deposited. After egg-laying, parents were placed in groups of four to five in a refrigerated aquarium (Dayno Mfg. Co., Lynn, Massachusetts) at 13°–15°C and fed tunicate viscera (*Ciona intestinalis*). Subsequent egg masses were assumed to have been fertilized predominantly by members of the group, all of which were then dissected and the statocyst condition recorded. This procedure was repeated three times so that about half the animals used in the present study were reared from egg masses deposited by parents of known statocyst type.

Animals were cultured in the laboratory through the planktonic veliger stage to the benthic juvenile and adult stages as previously described by Harrigan and Alkon (1978). All cultures were started from a mixture of veligers from at least 2–4 egg masses. Twenty percent of the individuals included in the present study were cultured in 5 μg/ml of the antibiotic chloramphenical (Sigma); the remainder were exposed to 5 μg/ml of rifampicin (Sigma). Unless otherwise noted, all animals were cultured under a 12 hour light/dark cycle at a light intensity of $3–5 \times 10^3$ ergs cm^{-2} s^{-1} and at 13°–15°C.

To determine if light intensity and photoperiod affected statocyst development, three egg masses deposited by wild specimens of *Hermissenda* were divided in half; each half was maintained from the two-cell stage through hatching in either constant light of 8×10^3 ergs cm^{-2} s^{-1} or 15 minutes light:23.75 hours dark. Larval and post-metamorphic stages were cultured in the same illumination condition experienced prior to hatching, and in 5 μg/ml rifampicin.

All post-metamorphic animals were maintained individually in 100 ml of standing seawater, with or without antibiotic, and changed three times per week initially and then daily when the animals reached 1 cm body length. Animals were fed an excess of tunicate viscera daily, so that growth rates were not food limited. At 30–50 days post-metamorphosis, adults were transferred to a slow flow-through seawater system (1 liter/min, 15°C), and kept individually on a light cycle of 6.5 hours light (at 6×10^3 ergs cm^{-2} s^{-1}):17.5 hours dark during behavioral training and testing.

Body length was measured from the anterior end of the animal, at a point halfway between the oral tentacles, to the tip of the tail when the animals were fully extended and moving forward (Risso-Dominguez, 1963). Photomicrographs and measurements of very small animals and statocysts were made through a Zeiss Universal microscope

with a calibrated ocular micrometer. Growth rates were compared using either Student's *t*-test (*t*) or analysis of variance (F) for unequal sample sizes (Snedecor and Cochran, 1967).

Electron microscopy

Animals were dissected and whole brain preparations were prepared according to previously described methods (Alkon, 1975). After the preparations were tested electrophysiologically the incubation medium was poured off and two to three drops of either of the following fixatives were placed onto the pinned brain preparations: 3% glutaraldehyde in either 0.1 *M* sodium cacodylate buffer (pH 7.3); 0.1 *M* sodium phosphate buffer plus 5 m*M* $MgCl_2$ and 5 m*M* EGTA (pH 7.4); 0.1 *M* HEPES buffer plus 5 m*M* $MgCl_2$ and 5 m*M* EGTA (pH 7.4). In all cases the osmotic pressure was adjusted to 1100 to 1200 milliosmoles with sucrose. After allowing the fixative to remain on the preparations for two to three minutes, the pins were removed and the entire brains placed in vials containing fresh solutions of their respective fixatives (30 min, 20°C). Following fixation the tissues were rinsed in their respective buffer compositions minus glutaraldehyde. In the final wash, the brains were bisected and all extraneous connectives removed. Post-fixation was carried out for 30 to 45 minutes (20°C) in a buffered 2% osmium tetroxide solution followed by straight buffer and distilled water washes before dehydrating in a graded ethanol series. Propylene oxide was used as a transitional solvent prior to infiltrating and embedding the tissue in an epon-araldite mixture (Geiselman and Burke, 1973). Hardened plastic blocks were subsequently sectioned with a diamond knife (Dupont Corp.) on an LKB 8800 Ultrotome III. Thick sections (1.0–2.0 μm) used for orientation and sensory cell counts were mounted on glass coverslips and stained with methylene blue or toluidine blue in 1% sodium borate (70°C). This sections were stained with aqueous uranyl acetate and lead citrate or vanadatomolybdate (Callahan and Hornes, 1964), and viewed on a Philips EM 300 or EM 400T transmission electron microscope. Live whole-mount brain preparations and sectioned material were observed and photographed on a Zeiss Universal microscope equipped with differential interference contrast optics.

Behavioral training and testing

The behavioral training apparatus and testing procedures for photopositive behavior have been described previously (Crow and Alkon, 1978; Tyndale and Crow, 1979; Crow, 1983, 1985; Harrigan and Alkon, 1985). Briefly, the time (latency) taken by laboratory-reared animals to move the length of 228 mm long seawater-filled glass tubes from very dim to brighter illumination (white light, 5.2×10^3 ergs cm^{-2} s^{-1}) was recorded automatically by photocells located beneath the glass tubes in the center of the turntable and connected to an event recorder. The glass tubes were attached to clips on a modified turntable so that one end, the start, was at the periphery (dim light) and the other end, the brightly illuminated area, faced the center of rotation. After two days of baseline testing to determine the latencies to move into the illuminated area the animals were placed on an automated training schedule consisting of 50 trials of diffuse light (30 s) paired with rotation (30 s at 2.24 g) of the turntable. After three consecutive days of training (total trials = 150) the animals' response latencies to enter the illuminated area were remeasured at the conclusion of the last training session and again at 24 and 48 hours.

It was previously shown for field-collected animals (Crow and Alkon, 1978; Farley and Alkon, 1982; Crow and Offenbach, 1983; Crow, 1983, 1985; Harrigan and Alkon,

1985) and for laboratory-reared animals (Crow and Harrigan, 1979) that response latencies of trained animals were significantly longer following training as compared with baseline tests and with various control groups used to assess non-associative contributions to the change in phototactic behavior.

Intracellular recordings

Recordings using standard intracellular techniques were made simultaneously from the type B photoreceptors and hair cells of isolated nervous system preparations from eleven of the laboratory-reared animals containing statocysts with either one, several (4–7), or a typical number of statoconia (150–200). Details of the dissection, isolation of the nervous systems, and recording methods have been reported elsewhere (Alkon, 1973, 1975). Rotation of the isolated nervous system while recording intracellularly from statocyst hair cells followed established experimental procedures (Alkon, 1975). Recordings were made about 24 hours after the last training session, and reflected the long-term associative effects of behavioral training.

Results

Effect of culture conditions on statocyst development

Statocysts form during intracapsular development, in the late trochophore and early veliger stages. Larval statocysts, which in newly hatched veligers measure 16.3 ± 1.1 μm diameter ($n = 20$) uniformly contain a single statoconium measuring 9.2 ± 0.7 μm diameter ($n = 20$). In 45% of the laboratory-reared animals included in the present study, a single statoconium was retained in the adult stage in both statocysts.

Antibiotics, either 5 μg/ml chloramphenicol or 5 μg/ml rifampicin, were routinely added to the culture water to increase percent survival of larvae and juveniles. Growth rate in the first month post-metamorphosis, when individuals (isolated) were maintained in standing seawater culture containing either antibiotic, was an important factor in predicting the number of statoconia in the adult statocyst, even though most adults were dissected at 50–90 days post-metamorphosis after two or more weeks in flowing seawater without antibiotic.

Growth is slow for the first two weeks post-metamorphosis, averaging 0.08 mm/day ($n = 10$), perhaps reflecting energy requirements for the major changes in body structure accompanying metamorphosis. Subsequent increase in body length is approximately constant, averaging 0.70 mm/day ($n = 10$), until about 90 days post-metamorphosis when some individuals become senescent. At one month post-metamorphosis mean individual body length was 2.45 cm (range = 0.48–4.45 cm, $n = 10$). Dissection of ten small wild individuals between 1.00 and 1.50 cm long, which by extrapolation from growth rates of laboratory-reared animals would be within one month post-metamorphosis, revealed only typical adult statocysts with no apparent reduction in number of statoconia. This observation suggests that in wild populations statoconia formation is maximal in the first month post-metamorphosis.

Although the parents of half the laboratory-reared population of *Hermissenda* were dissected and found to contain typical statocysts, there was considerable variation in number of statoconia among their first-generation offspring. Growth rates (in mm/day) were compared, in the presence or absence of antibiotic, for animals with a single statoconium, several statoconia (including any case in which different numbers of stones were found in the paired statocysts), or with the typical number of statoconia per statocyst (Table I). Twelve of the 32 individuals with a single statoconium in the 'antibiotic' treatment were exposed to chloramphenicol and the rest to rifampicin.

TABLE I

Growth rates in first month post-metamorphosis

	No Antibiotic			Antibiotic		
	n	mm/day	Percent of total	n	mm/day	Percent of total
1. Single stone	3	0.63 ± 0.09	27	32	0.65 ± 0.27	48
2. Several stones	3	0.63 ± 0.28	27	23	0.81 ± 0.26	35
3. Typical	5	0.85 ± 0.08	46	11	0.97 ± 0.31	17
Total:	11			66		

Because there was no significant difference between their growth rates ($t_{30} = 0.631$, $P > 0.05$) the two samples were combined.

Table I illustrates the following points. First, in both the 'antibiotic' and 'no antibiotic' treatments animals with slower growth rates (<0.80 mm/day) retain the single statoconium condition as adults, whereas faster-growing animals (>0.80 mm/day) tend to have a typical complement of statoconia. Second, mean growth rates of antibiotic-treated animals, when separated into categories consisting of those with one, several, or a typical number of statoconia per statocyst, were overall significantly different ($F_{2,73} = 6.30$, $P < 0.01$). The Newman-Keuls test for ordered means showed a significant difference ($P < 0.05$) only between the mean growth rates for animals with a single statoconium and animals with typical statocysts. Mean growth rates for animals in the 'several' category were not significantly different from those of animals in the other two categories. Growth rates of animals with a single statoconium in the 'no antibiotic' treatment were significantly different from those of animals with typical statocysts ($t' = 3.50$, df = 4, $P < 0.05$). Third, a higher proportion (46%) of animals in the small 'no antibiotic' treatment grew relatively faster and had typical statocysts than in the 'antibiotic' treatment (17% with typical statocysts).

These data support the conclusion that exposure to either chloramphenicol or rifampicin at low concentrations (5 μg/ml) retards the growth rate to a variable extent in different individuals. There is also a significant positive relationship between the slowest growth rates and the retention of a single statoconium in the adult stage.

When animals from parents with typical statocysts, cultured from fertilization to maturity either in constant light of 8×10^3 ergs cm^{-2} s^{-1} (n = 7) or 15 minutes of room light per day (n = 4), were dissected, all were found to possess a single statoconium. Growth rates in the first month post-metamorphosis were relatively slow in each illumination condition, and corresponded to rates associated with the single statoconium condition in animals reared in 12 hours light/dark (LL = 0.40 ± 0.18 mm/day; 15 min L = 0.59 ± 0.23 mm/day).

Statocyst morphology

Animals with reduced numbers of statoconia or a single statoconium showed the same general statocyst morphology as animals with the standard complement. Cell counts done on stained thick sections (1–2 μm) revealed the usual number of 13 sensory (hair) cells. Interspersed between the sensory cells are the non-sensory support cells. The sensory kinocilia (hairs) had the same typical length (8–10 μm) and breadth (0.3 μm) found in statocysts containing the typical number of statoconia (150–200). Exact numbers of cilia/cell were not determined but the average spacing between cilia

was 1.4 μg. The ciliary morphology appeared within normal limits of that seen in the field-collected animals, including the basal bodies with their elaborate rootlet system located just below the apical surface of the sensory cell (Kuzirian *et al.*, 1981). The basic histology of the cytoplasm and organelles of both the sensory and support cells was also unremarkable (see Figs. 1–4).

The principal pathology demonstrated in statocysts from animals (n = 6) reared under laboratory conditions was dimensional. Statocysts containing reduced numbers of statoconia or a single statoconium were smaller. They averaged 40 to 55 μm in overall external diameter (90%: 45–50 μm, fixed preparations) which is approximately half the normal size. Concomitant with the overall size reduction, the lumenal diameter was reduced by four to five times, depending upon the number of statoconia present (10–25 μm, fixed preparations). The sensory cells, rather than being typically flattened (5–10 μm thick) with large, elongate nuclei, were 2.0 to 2.5 times thicker (13–17 μm) and possessed large oval to round nuclei. This increase in thickness was due solely to an increase in the amount of granuloplasm present within each sensory cell (*cf.* Geuze, 1968; Stahlschmidt and Wolff, 1972). Spacing between the sensory cilia (1.4 μm) was only half the distance found in typically developed statocysts. Observations made on live dissected preparations using differential interference contrast (Nomarski) optics indicated a reduced and/or restricted movement of the statoconia due to the small size of the lumen.

The reduced movement of the stones could also be due to the presence of an intraluminal matrix which was found in some specimens (Fig. 5). This organic matrix completely surrounded the stones. Additionally, the dense protein matrix layers normally composing the stones found in field-collected animals (Fig. 5, insert) is greatly reduced, much less dense and very loosely organized, or in some stones almost entirely absent. The interluminal protein matrix coupled with the lack of organization of the intra-statolith protein matrix may be indicators of a major perturbation of normal protein chemistry brought about by laboratory culture conditions and the principal suspected cause of the observed pathology.

Behavior

Following measurement of phototactic behavior after behavioral training (see Materials and Methods), the laboratory-reared specimens of *Hermissenda* were dissected and the two statocysts examined. Animals were divided into groups, those with several or a typical number of statoconia and animals with one statoconium. Dissections were done blind so that the experimenter did not know the results of the behavioral tests for the individual animals.

There were no significant differences in behavior before or after training for animals from the 'no antibiotic' and 'antibiotic' groups. However, in contrast to the 'antibiotic' treatment, there was a significant difference in the latencies to enter light between animals with a single statoconium and those with several to typical numbers of statoconia (Mann-Whitney U-test, n = 19, $P < 0.02$; Hollander and Wolfe, 1973). The response latencies before training were not different for animals with variable numbers of statoconia.

Laboratory-reared specimens of *Hermissenda* with a single statoconium exhibited faster response latencies following training as compared to animals with a typical or reduced complement of statoconia (Fig. 6). The reduced effectiveness of the training procedure for *Hermissenda* specimens containing a single statoconium is consistent with observations made on field-collected animals that had statocysts containing a single statoconium (Crow and Harrigan, 1979).

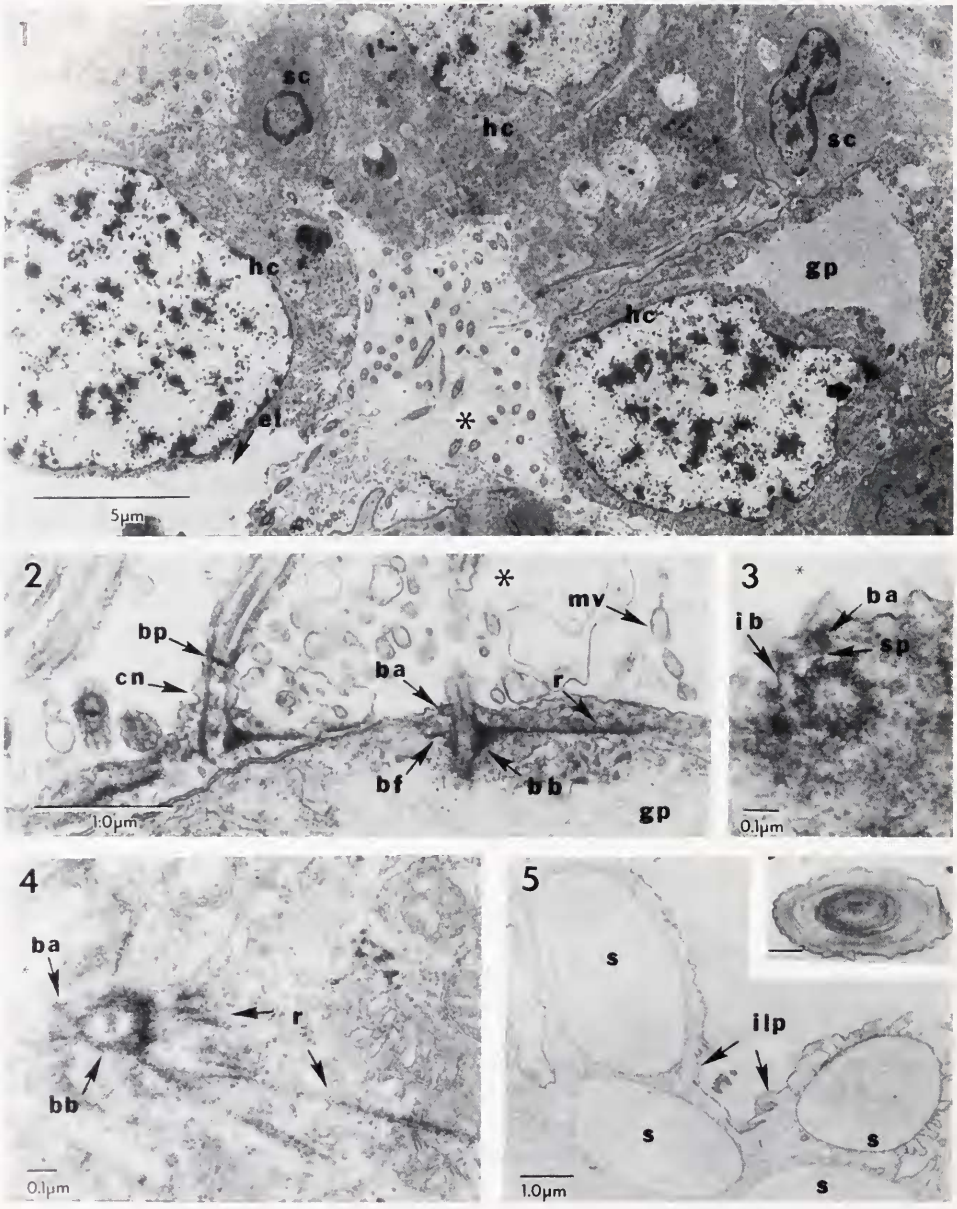

FIGURE 1. Low-power electron micrograph of statocyst from a laboratory-reared animal illustrating the anomalous morphology of the (1) thickened sensory hair cells (hc) with their large round nuclei and in the cell at the right, a portion of the increased amount of granuloplasm (gp) and (2) the reduced size of lumen (*) into which the sensory cilia project: el, electrode lesion, indicating penetration site of microelectrode used for electrophysiologic testing (basement membrane removed by protease digestion to facilitate microelectrode penetration).

FIGURE 2. Longitudinal section of two kinocilia from two adjacent hair cells demonstrating the normally expected appearance: ba, button anchor; bb, basal body; bf, basal foot; bp, double basal plate indicating termination point of central axoneme; cn, ciliary necklace region; gp, granuloplasm of hair cell; *, statocyst lumen; mv, microvillus, projecting from hair cell's apical surface; r, laterally projecting rootlet system.

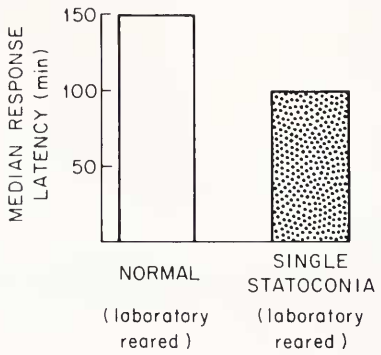

FIGURE 6. Median response latencies of laboratory-reared *Hermissenda* individuals with a normal complement of statoconia compared to those with single statoconia tested immediately following three days of training.

These results from laboratory-reared animals with a single statoconium may help to elucidate the involvement of the statocysts in the modification of phototactic behavior since the contribution of non-specific sensory input resulting from rotation would not be expected to change in these preparations.

Electrophysiology

Statocyst hair cells and photoreceptor cells synapse directly onto each other and also converge onto interneurons in the optic and cerebropleural ganglia (Tabata and Alkon, 1982; Alkon, 1983; Fig. 7). Intracellular recordings from caudal hair cells and/or ipsilateral Type B photoreceptors were obtained from the isolated circumesophageal nervous systems of *Hermissenda* (Alkon, 1975). The isolated nervous systems were oriented with respect to the center of rotation such that rotation caused the statoconia, as they experience angular acceleration, to exert pressure on the hairs of the caudal cells in the two statocysts. For statocysts with a typical number (150–200) of statoconia, rotation results in a depolarizing generator potential and an increase in spike frequency recorded from the cell bodies of caudal hair cells as previously reported (Alkon, 1975). When the response to rotation is recorded from a simultaneous penetration of a caudal hair cell and an ipsilateral Type B photoreceptor, the response recorded from both cells is a graded function of the number of statoconia (Fig. 8A, B, C). In statocysts with only a single statoconium the depolarization in response to rotation ($\leq$0.9 *g*) was greatly reduced or did not occur (Fig. 8C; 9A; Table II). As

FIGURE 3. Tangential section just below hair cell's apical surface illustrating four of the nine button anchors (ba) with their spoke connections (sp) to the triplet tubules of the basal body as well as the osmiophillic interconnecting bridges (ib) between them (*, statocyst lumen).

FIGURE 4. Slightly oblique section through the hair cell's apical surface demonstrating the asymmetrical elaboration of the basal body (bb) rootlet system (r) in a form resembling an astral array; ba, button anchor with spoke connection to basal body (*, statocyst lumen).

FIGURE 5. Micrograph of several cross-sectioned statoconia (s) from a statocyst containing a greatly reduced number of stones illustrating intraluminal protein matrix (ilp) binding stones together and demonstrating the lack of density and organization of the protein matrix typically seen in statoconia of field-collected animals (insert: scale bar = 1.0 μm).

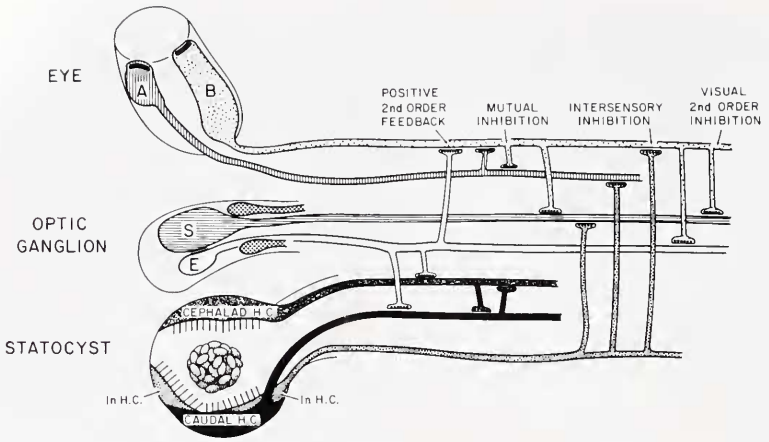

FIGURE 7. Schematic diagram of connections between the visual and statocyst pathways, as determined from intracellular recordings. Note that sensory cell interactions are primarily mutually inhibitory. Caudal hair cells synaptically inhibit the type B photoreceptor; type B cell inhibits second-order visual cells in the optic ganglion, resulting in excitation of the cephalad hair cell and inhibition of the caudal hair cell. A, B: type A and type B photoreceptor cells. S, E: optic ganglion cells. H.C.: hair cell (from Alkon, 1979).

statoconia number increased above three, the amplitude of the depolarizing response evoked by rotation was increased (Fig. 8B).

With the same orientation of the nervous systems as just described (the caudal orientation) Type B photoreceptors in preparations with typical statoconia number usually hyperpolarize in response to rotation (Fig. 8A; 9B; also, Tabata and Alkon, 1982). For Type B cells from preparations having statocysts with only a single statoconium, this hyperpolarizing response to rotation (≤ 0.9 *g*) was absent (Fig. 8C, upper trace; Table II). As statoconia number increased above three, the hyperpolarizing response of the ipsilateral Type B photoreceptors was evident although not of the same magnitude as the response from typical statocysts (see Fig. 8A, B). Simultaneous recordings from type B cells and caudal hair cells containing eight or more statoconia could not be distinguished from those obtained from field-collected animals, indicating that hair cell-photoreceptor synapses function normally in laboratory-reared animals with several or many statoconia. The degree of synaptic inhibition between caudal hair cells and ipsilateral type B cells was similar in all preparations and independent of statoconium number.

Tabata and Alkon (1982) recorded from 14 caudal hair cell-Type B photoreceptor pairs from animals with typical statocysts; in these cases B-cell inhibition occurred in 8 out of 14 pairs. Synaptic inhibition of Type B cells by caudal hair cells can account, therefore, for the hyperpolarizing response of Type B cells to rotation (*i.e.*, in the caudal orientation; Fig. 9B). However, a change in this typical synaptic interaction cannot account for the absence of Type B hyperpolarizing responses observed from preparations with single statoconium statocysts (Fig. 8C; 9A). With single statoconia, synaptic inhibition of Type B cells by caudal (ipsilateral) hair cells was readily demonstrated (Fig. 9C; Table II, 3 out of 4 preparations). This suggests that the absence of a hyperpolarizing response in the B photoreceptor as evoked by rotation is due to the ineffectiveness of the *g* forces used to produce a depolarizing generator potential in studies of these nervous systems.

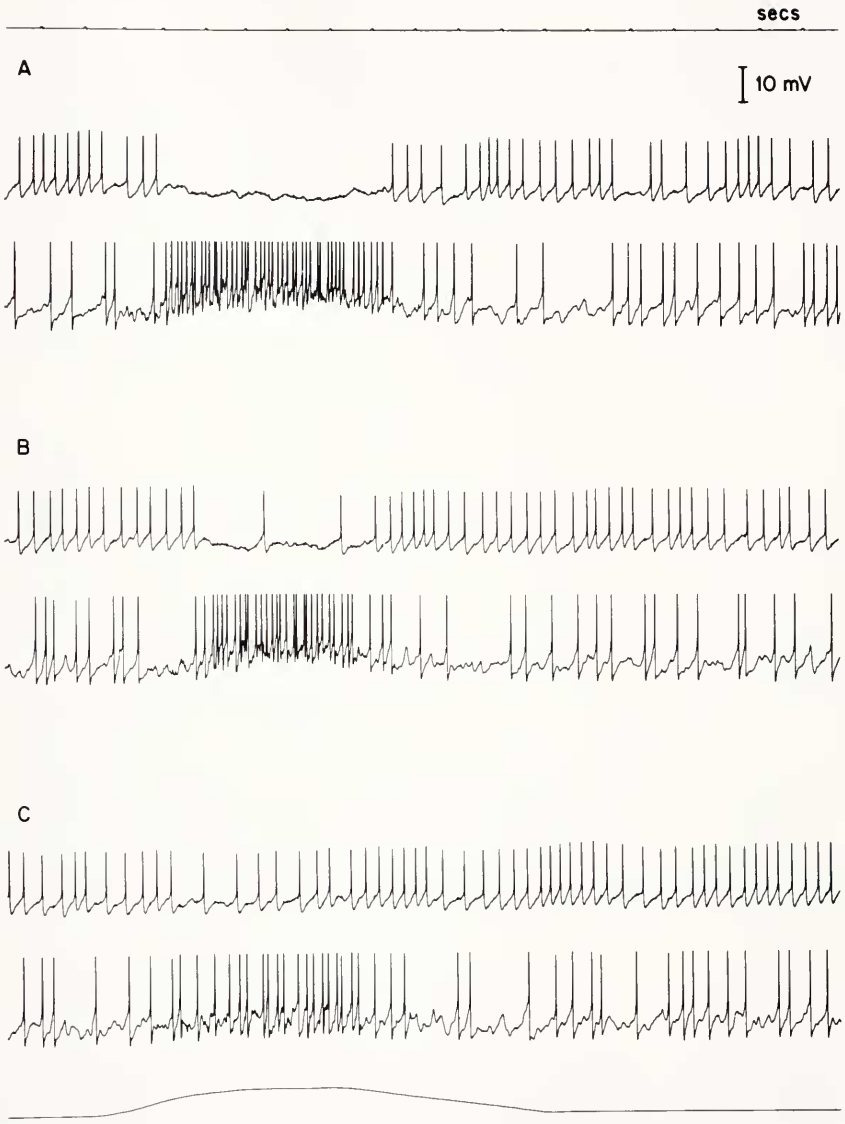

FIGURE 8. Intracellular recordings from type B ipsilateral photoreceptors (upper traces) and caudal hair cells (lower traces). Single trace at the bottom illustrates the time course of the rotational stimulus. A. Preparation containing typical complement (150–200) statoconia. B. Preparation with several statoconia. C. Preparation containing a single statoconium.

Together, these results clearly indicate that for single statoconium preparations, Type B cells do not hyperpolarize in response to rotation (≤ 0.9 *g*) because ipsilateral caudal hair cells do not show their normal depolarizing responses. Presumably with somewhat higher *g* force such as that used in training (2.24 *g*) caudal hair cells from statocysts with a single statoconium would show some depolarizing response, albeit of much smaller than normal magnitudes.

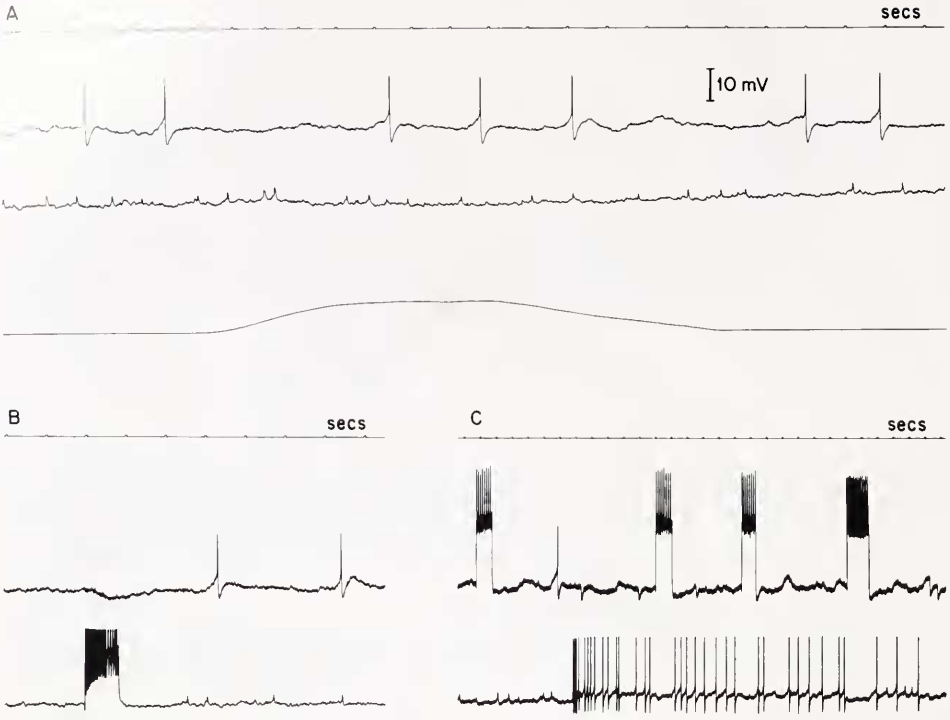

FIGURE 9. A. Intracellular recording from Type B photoreceptor (upper trace) and caudal hair cell (lower trace) in a preparation containing a single statoconium. Single trace at the bottom illustrates the time course of the rotational stimulus demonstrating the absence of a generator potential in response to rotation ($g = 0.9$). B. Synaptic inhibition and hyperpolarization of Type B photoreceptors resulting from an electrically produced firing burst in a caudal hair cell preparation with typical number of statoconia. C. Synaptic inhibition of a Type B photoreceptor resulting from electrical stimulation of an ipsilateral caudal hair cell in a statocyst containing a single statoconium.

DISCUSSION

Although antibiotics are used routinely to control bacterial populations in marine invertebrate culture systems (Smith and Chanley, 1972), little is known about their physiological effect on growth and morphology of the developing animal. Biochemical investigations have shown that both chloramphenicol and rifampicin inhibit protein synthesis in eukaryotes (Corcoran and Hahn, 1975). These antibiotics, used in the present study at 5 μg/ml each, inhibited the post-metamorphic growth rate of *Hermissenda* relative to growth rates in the absence of antibiotic. Both antibiotics were applied within the reported concentration range for effects on eukaryotic systems (Corcoran and Hahn, 1975).

Antibiotic-induced inhibition of growth rate was the only variable that was significantly related to the number of statoconia per statocyst. With few exceptions, the slowest-growing animals possessed statocysts containing a single statoconium. Because three out of eleven animals maintained in the absence of antibiotics contained a single statoconium in each statocyst, and because a similar sample of very small (1.0–1.5

TABLE II

Responsiveness of Type B photoreceptors and caudal hair cells to rotation (0.4, 0.5, 0.6 g) or to reciprocal stimulation in preparations with 1, several, or > 8 statoconia

	0 = No response				1 = Response				± = Threshold response	
	Type B hyperpolarization				Caudal hair cell depolarization					
	0.4	0.5	0.6	g	0.4	0.5	0.6	g	H.C. → B.	B. → H.C.
Single statoconium										
1.	0	0	0		0	0	0		0	0
2.	0	0	0		0	±	±		1	1
3.	0	0	±		0	±	±		1	1
4.	—	—	—		0	0	0		—	—
5.	0	0	0		0	0	0		1	1
3–8 statoconia										
6.	0	±	±		0	0	±		1	0
7.	1	1	1		1	1	1		1	1
8.	0	0	0		1	1	1		0	—
9.	0	0	0		—	—	—		0	0
10.	0	0	0		1	1	1		—	—
>8 statoconia										
11.	1	1	1		—	—	—		0	1
12.	1	1	1		1	1	1		1	0
13.	—	—	—		1	1	1		—	—

H.C. = Hair cell.
B = type B photoreceptor.

cm) wild *Hermissenda* uniformly contained typical statocysts, other variables such as maintenance in standing *versus* flow-through sea water, diet, and possibly effects of early experience of mechanical stimulation must affect statoconium replication, especially directly after metamorphosis, and need to be investigated.

Schmidt (1912), and McClary (1963) have postulated intraluminal crystallization of stones from a $CaCO_2$-organic fluid matrix. This process may be interfered with by the laboratory culture conditions and thus directly limit statoconia production by preventing the crystallization process rather than interfering with the intracellular production of their components. However, in a recent study of field-collected adult *Hermissenda,* the evidence supported the hypothesis that statoconia production occurred intracellularly within large coated vesicles (Kuzirian *et al.,* 1981). Evidence for the intracellular production of statoliths was also reported by Geuze (1968) for the basomatophoran pulmonate *Lymnaea stagnalis.* Thus, the spurious intraluminal protein matrix is totally artifactual and probably directly related to culture conditions.

Previous studies have often utilized statocyst extirpation experiments as a means of assessing the contribution of the statocyst pathway to the orientation of marine invertebrates, especially crustaceans, in which the statocysts are often located in the bases of the easily removable antennules (Creutzberg, 1975). Similar experiments in a soft-bodied animal such as *Hermissenda,* in which the statocysts are in close contact with the cerebropleural ganglion and are only visible upon dissection, are not readily feasible. Therefore, the discovery that animals possessing either one, several, or a

typical complement of statoconia could be reliably produced provides a useful tool for analyzing the contribution of the statocyst pathway to the behavioral change resulting from stimulation with paired light and rotation. The contribution of nonspecific stimulation resulting in arousal could not be expected to change in these preparations. Therefore, changes in the efficacy of the associative effect indicate that the statocyst pathway is important in mediating this example of associative learning.

The nature of mechanotransduction by the sensory cilia has been investigated in both *Hermissenda* (Alkon, 1975; DeFelice and Alkon, 1977; Grossman *et al.*, 1979; Stommel *et al.*, 1980; Grossman and Alkon, 1983) and *Aplysia* (Gallin and Wiederholt, 1977). Grossman *et al.* (1979) found that voltage noise variance of hair cells with cut axons increased with increasing depolarization when the isolated nervous system was rotated at accelerations from 0.5 to 2.0 *g*. Hair cells with unloaded hairs (opposite to the mass of statoconia) had low noise levels during rotation, although the hairs continued to beat. The authors concluded that both the voltage noise and the generator potential originate from contact between the statoconia and the motile cilia. Evidence from voltage noise power spectra recorded from *Hermissenda* hair cells also indicates that most noise arises from statoconia-ciliary interactions (Grossman and Alkon, 1983). Recordings from hair cells of a statocyst containing a single statoconium from a wild *Hermissenda* showed a smaller voltage noise variance than those from a typical statocyst (DeFelice and Alkon, 1977). The size of the generator potential can be considered to reflect the average value of all the conductance changes initiated as a function of stimulus strength, in turn a function of the number of statoconia as well as the acceleration of gravity (*g*-value) (DeFelice and Alkon, 1977).

Stommel *et al.* (1980) studied the mechanism of sensory transduction in the *Hermissenda* statocyst by modifying the pattern of ciliary beat. Intracellular injection of vanadate anion was followed by a decrease, then cessation of voltage noise paralleling progressive ciliary relaxation, then loss of motility. Injection of 4,4′-dithiobisphenylazide caused the cilia to stiffen, with an increase in voltage noise amplitude if the cilia were beating, and minimal noise when the beat was inhibited. The authors suggest that mechanical stimuli are transduced at the junction between the ciliary membrane and the plasma membrane, where a beating cilium causes flexing of the adjacent plasma membrane.

In intact *Hermissenda,* the less effective transduction of hair cells when only one statoconium is present provides a functional explanation for the significantly reduced effectiveness of training in these animals. Any factor, such as reduction of statoconium number, that decreases the effect of rotation on hair cell depolarization would also be expected to decrease the amount of inhibition received by the photoreceptors, whether directly through possible monosynaptic connections or by way of projections onto the optic ganglion cells.

Details of the circuitry by which a decrease in hair cell generator potential and spike frequency causes a decreased effectiveness of training with paired light and rotational stimulation have been proposed (see Fig. 7 and Tabata and Alkon, 1982). Because multiple pathways allow feedback from interneurons in the optic and cerebropleural ganglia onto hair and photoreceptor cells, we cannot rule out effects of activity in other circuits on acquisition and retention of behavioral conditioning. The only observable morphological variable in this study that might affect caudal hair cell function was reduction in statoconium number and reduced lumenal surface area. Based on present knowledge, it seems reasonable that as a result of decreased synaptic output due to less rotation-induced caudal hair cell stimulation, less depolarization of the type B photoreceptor follows pairings of light and rotation (Alkon, 1984).

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